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Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo

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Abstract

ALDP, ALDPR, PMP70 and PMP70R are half ATP-binding cassette (ABC) transporters of the mammalian peroxisomal membrane. By analogy with other members of this family, it is assumed that peroxisomal ABC transporters must dimerize to become functional units. However, not much is known regarding the type of dimers (i.e., homodimers and/or heterodimers) that are formed in vivo under normal expression conditions. In this work, we have characterized the quaternary structure of mouse liver PMP70 and ALDP. The PMP70 protein complex was purified to apparent homogeneity using a two-step purification protocol. The ALDP-containing protein complex was characterized by preparative immunoprecipitation experiments. In both cases, no evidence for the existence of heteromeric interactions or for the presence of accessory proteins in these ABC transporter protein complexes could be obtained. Our data indicate that the majority (if not all) of mouse liver PMP70 and ALDP are homomeric proteins.

Keywords: Half ABC transporter; Peroxisome; Adrenoleukodystrophy; X-ALD; Membrane protein purification

1. Introduction

The ATP-binding cassette transporters (ABC transporters) represent one of the largest families of proteins, with 48 ABC transporters already identified in human. These proteins are molecular pumps that couple ATP hydrolysis to the transport of substrates (e.g., cholesterol, bile salts, polypeptides, iron) across biological membranes (reviewed in Ref. [1]). Structurally, the members of this family have two transmembrane domains, each comprising several α-helices, and two conserved nucleotide binding domains (reviewed in Ref. [2]). In eukaryotes, the ABC proteins are classified either as full transporters, if this core of four domains is encoded by only one gene (e.g., CFTR), or as half-transporters, if the corresponding genes encode only one transmembrane domain and one nucleotide binding domain (e.g., Tap1 and Tap2). The latter class of proteins becomes functional only after dimerization (reviewed in Ref. [3]).

In mammals, four half ABC transporters are present in the peroxisomal membrane: the adrenoleukodystrophy protein (ALDP or *ABCD1* [4]), the ALDP-related protein (ALDPR or *ABCD2* [5]), the peroxisomal membrane protein of 70 kDa (PMP70 or *ABCD3* [6]) and the PMP70-related protein (PMP70R or *ABCD4* [7,8]). By analogy with other members of the half ABC transporter family (e.g., Tap1 and Tap2), it has been assumed for long that these peroxisomal transporters must dimerize to become functional units.

Abbreviations: ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; ALDPR, ALDP-related protein; PMP70, peroxisomal membrane protein of 70 kDa; PMP70R, PMP70-related protein; X-ALD, X-linked adrenoleukodystrophy; ESI-MS, electrospray ionization mass spectrometry

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Whether dimerization results in the formation of homodimers only or if heterodimers can also be formed is an issue that did not receive much attention up to now. In fact, although all these peroxisomal proteins were identified several years ago, only three studies have addressed this problem. In one of these studies, yeast two-hybrid experiments together with Western blotting analysis of immunoprecipitates obtained from cultured cells overexpressing peroxisomal transporters led to the idea that ALDP, ALDPR and PMP70 are quite promiscuous entities capable of engaging in both homo- and heterodimerization processes [9]. However, due to the nature of the experimental techniques used in that study, it is not evident what kind of interactions prevails in vivo (i.e., under normal expression conditions of the peroxisomal transporters). Exactly the same remark can be made regarding the in vitro experiments described by Smith et al. [10]. In that work, a co-immunoprecipitation assay employing in vitro synthesized proteins was used to identify protein–protein interactions involving ALDP, PMP70 and ALDPR. Finally, in the third study addressing this issue, purified peroxisomes from rat liver were used in immunoprecipitation experiments. The authors were able to demonstrate co-immunoprecipitation of ALDP with PMP70 [11]. However, several other peroxisomal proteins were also found in the immunoprecipitates. Thus, it is not clear whether the PMP70–ALDP interaction is direct or indirect.

Defining the kind of protein interactions in which peroxisomal transporters are involved in vivo is an important task having major implications on our knowledge on the biochemistry of the mammalian peroxisome. If it is assumed that peroxisomal transporters exist in the peroxisomal membrane solely as homodimers, then only four different substrates (or families of substrates) are expected to be transported by these proteins. If, however, peroxisomal transporters are promiscuous entities capable of interacting with each other, then 10 different combinations of transporters can be formed raising the possibility that 10 different substrates (or families of substrates) use these transporters to cross the peroxisomal membrane. In the particular case of ALDP, it is likely that such information is of fundamental importance to understand the pathogenesis mechanism in X-linked adrenoleukodystrophy (X-ALD), a human genetic disease caused by mutations in the ALDP gene (reviewed in Ref. [12]) and that affects 1:21,000–1:100,000 males [13,14].

Although the exact function of ALDP is not presently known, it has been proposed that it transports very long chain fatty acids (VLCFAs) into the peroxisomal compartment or some factor necessary for the peroxisomal β-oxidation of these compounds [4,15]. This conclusion derives from the fact that X-ALD patients accumulate VLCFAs in many organs (liver included [16]) and body fluids (reviewed in Ref. [12]), a biochemical alteration that was also observed in several ALD knockout mice [16–18]. However, the correlation between accumulation of VLCFAs and clinical phenotype in X-ALD is far from being clear [19], raising the possibility that other (still unknown) biochemical factors contribute to the pathology. To define the biochemical role of ALDP, it is of the utmost importance to know the kind of protein–protein interactions in which ALDP is involved in vivo. If ALDP is a homodimeric protein, then only one primary biochemical defect should occur in the absence of a functional protein. If, however, ALDP is capable of forming homo- and heterodimers, then it is plausible to assume that the metabolism of more than one type of substrates will be affected by the absence of functional ALDP.

In this work, we have characterized the quaternary structure of mouse liver PMP70 and ALDP. After solubilization of peroxisomal proteins using the mild detergent digitonin, a simple two-step procedure was used to purify PMP70. The ALDP protein complex was characterized by preparative immunoprecipitation experiments. Our results indicate that both half ABC transporters exist in the peroxisomal membrane predominantly (if not exclusively) as homomeric proteins, probably dimers.

2. Materials and methods

2.1. Purification of mouse liver peroxisomes

Peroxisomes were isolated from the liver of C57/BL6 male mice (1–3 months of age) by differential centrifugation and purified through a Nycodenz gradient essentially as described [20,21]. The Nycodenz gradients (6 ml of 30% and 2 ml of 25% (w/v) Nycodenz in 5 mM imidazole–HCl, pH 7.4 and 1 mM EDTA–NaOH, pH 7.4) were centrifuged in the fixed-angle rotor T1270 (Sorvall, Ultra Pro80 centrifuge) at $60\,000 \times g$ for 30 min. Peroxisomes obtained in this way were estimated to be 90% pure presenting a minor contamination with mitochondria and endoplasmic reticulum.

2.2. Solubilization of peroxisomal proteins

In a typical experiment, 1 mg of purified peroxisomes were incubated in 500 μl of solubilization buffer (see bellow) for 30 min at 4 °C with gentle agitation. Protein samples were centrifuged at $105\,000 \times g$ for 1 h at 4 °C using the rotor T1270. To determine yields of solubilization, equivalent portions of supernatants and pellets were subjected to Western blotting analysis using antibodies directed to mouse peroxisomal ABC transporters. Good solubilization yields (approximately 80%) were obtained when using a buffer (hereafter referred to as buffer A) containing 1% (w/v) digitonin [added from a 5% (w/v) stock solution prepared as suggested by the manufacturer (Calbiochem)], 50 mM Tris–acetic acid, pH 7.5, 0.1 M potassium acetate-acetic acid, pH 7.4, 0.25 M 6-aminocaproic acid, 1 mM EDTA–NaOH, pH 7.4, 1:500 (v/v)

protease inhibitor cocktail (Sigma P8340) and 0.1 mg/ml phenylmethylsulfonyl fluoride.

2.3. Sucrose density gradient analysis

Detergent-solubilized proteins (2 mg of protein in 1 ml of buffer A) were loaded onto the top of a discontinuous sucrose gradient [2 ml of 10%, 1.8 ml of 15.5%, 1.7 ml of 21%, 1.5 ml of 25%, 1.2 ml of 30%, 1.0 ml of 35% and 0.3 ml of 40% (w/v) sucrose in a buffer containing 50 mM Tris–acetic acid, pH 7.5, 10 mM potassium acetate–acetic acid, pH 7.4, 1 mM EDTA–NaOH pH 7.4 and 0.1% (w/v) digitonin]. In the experiment shown in Fig. 1, centrifugation of the sucrose gradients was performed in the swinging bucket rotor TST 41.14 rotor (Sorvall, OTD75B centrifuge) at 160 000 × *g* for 16 h at 4 °C. In Fig. 2, the sucrose gradient was centrifuged at 180 000 × *g* for 16 h at 4 °C using the swinging bucket rotor TH-641 (Sorvall, Ultra Pro80 centrifuge). Twelve fractions of 875 μl were collected from the bottom of the tube and subjected to precipitation with 10% (w/v) trichloroacetic acid.

Treatment with urea of digitonin-solubilized peroxisomal proteins was done as follows: 200 μg of purified peroxisomes were incubated with 50 μl of buffer A for 15 min at 4 °C with gentle agitation. After adding 25 μl of 9 M urea (final concentration of 3 M), protein samples were incubated 15 min at room temperature, diluted with 925 μl of buffer A and loaded onto the top of a sucrose gradient.

2.4. Purification of mouse liver PMP70 complex

The purification protocol was started with a pellet of 3.3 mg of purified peroxisomes. The organelles were solubilized for 30 min at 4 °C, in 550 μl of buffer B (buffer A without EDTA, plus 2 mM $MgCl_2$ and 0.5 mM $MnCl_2$). The sample was subjected to centrifugation at 105 000 × *g* for 1 h at 4 °C to remove insoluble material. The supernatant was added to 150 μl (bed volume) of ATP-agarose (C-8 attachment; Sigma) previously equilibrated in buffer C (buffer B containing 0.1% (w/v) digitonin). After incubation for 1 h at 4 °C in an end-over-end shaker, the ATP-agarose slurry was washed twice with 1.5 ml of buffer C supplemented with 5 mM $MgCl_2$ (final concentration). The proteins were then eluted with 600 μl of buffer C containing 10 mM $MgCl_2$ and 10 mM ATP for 1 h at 4 °C with occasional shaking. Eluted proteins were loaded onto the top of a sucrose gradient (rotor TH-641), centrifuged and fractionated, as described above.

2.5. Immunoprecipitation of mouse liver ALDP

Two milligrams of purified peroxisomes were solubilized in 2 ml of buffer A containing 5 mM iodoacetamide for 30 min at 4 °C and subjected to centrifugation (105 000 × *g* for 1 h at 4 °C) to remove insoluble material. One aliquot (220 μl) of the supernatant (sample "T") was kept on ice. The remaining supernatant was halved. Each half was added to

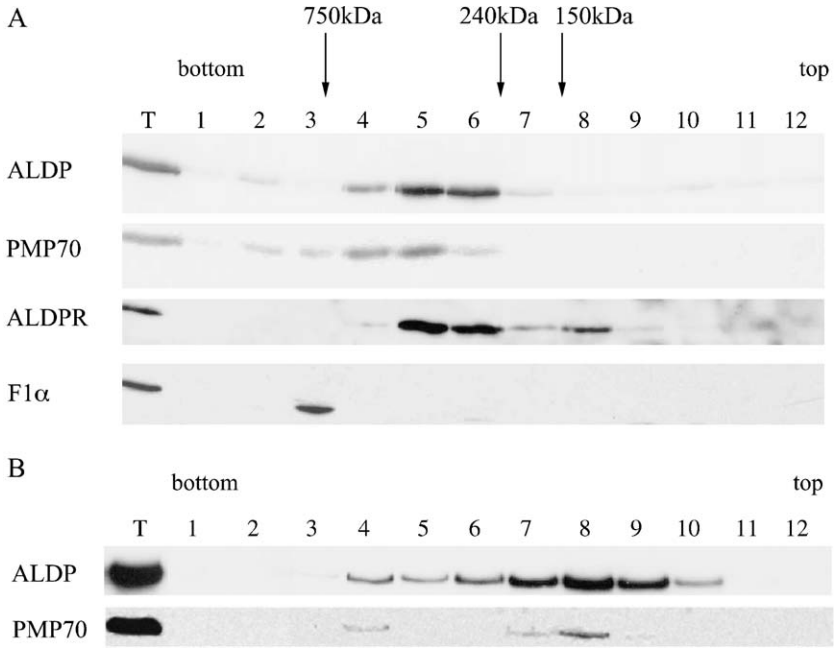
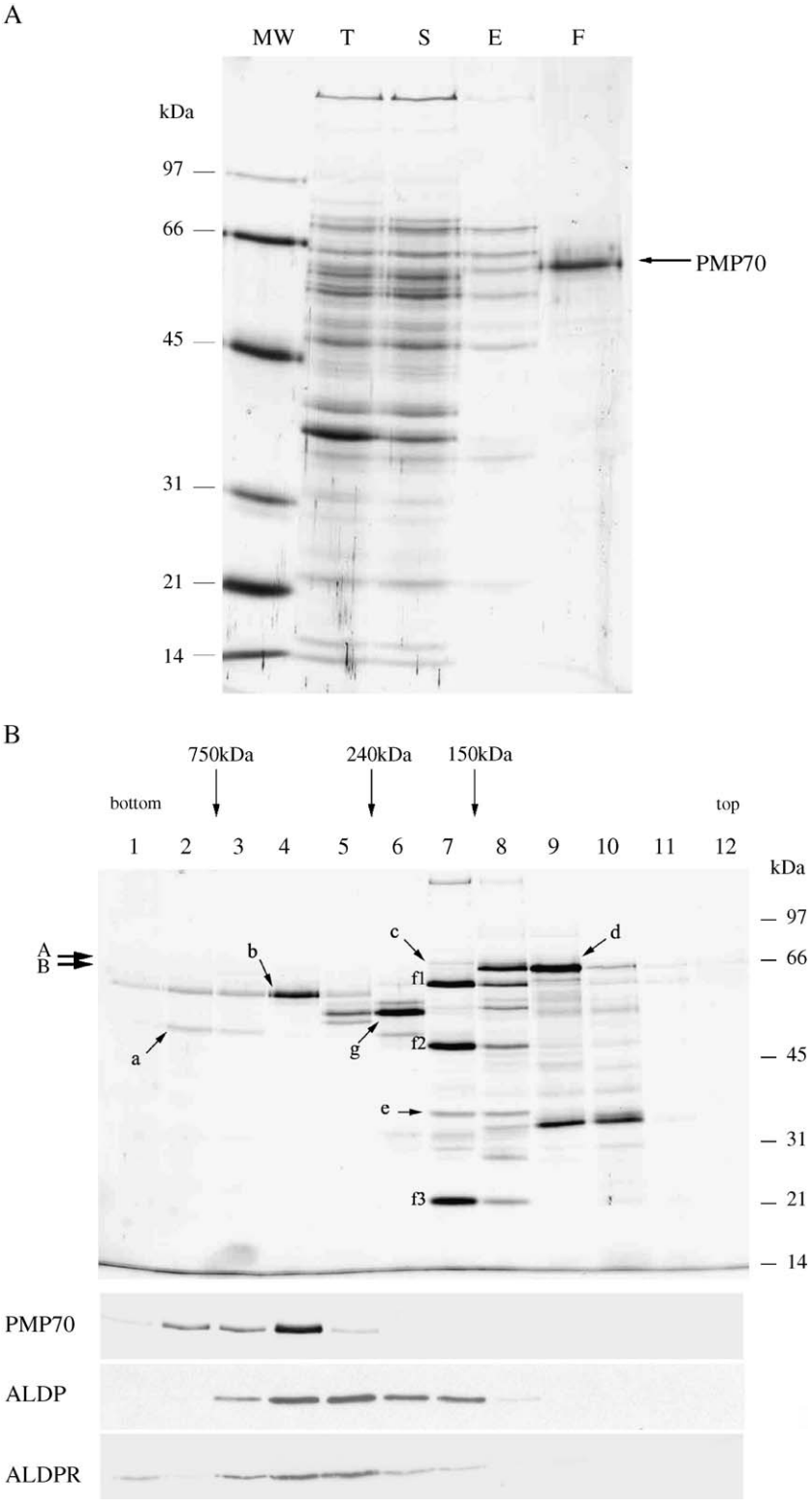


Fig. 1. Sedimentation analysis of digitonin-solubilized mouse liver ALDP, PMP70 and ALDPR. (A) Digitonin-solubilized peroxisomal proteins were loaded onto the top of a sucrose gradient. After centrifugation, the gradient was fractionated starting from the bottom (lane 1) to the top (lane 12). Equivalent portions of each fraction (corresponding to 210 μg of peroxisomal protein) were precipitated with trichloroacetic acid and analyzed by immunoblotting using antibodies directed to ALDP, PMP70, ALDPR and the α subunit (F1α) of the mitochondrial ATPase complex (molecular mass of 750 kDa). The positions of catalase (240 kDa) and palmitoyl-CoA oxidase (150 kDa) in the gradient are also indicated. Lane T, 70 μg of peroxisomal protein. (B) Digitonin-solubilized peroxisomal proteins (200 μg) were incubated in the presence of 3 M urea, subjected to sucrose gradient centrifugation and analyzed as described above.

12 mg of CNBr-activated Sepharose 4B beads that had been previously coupled with anti-ALDP or pre-immune immunoglobulins G (IgGs; see below). The samples were incubated for 3 h at 4 °C, in an end-over-end shaker. The beads were collected by centrifugation (10 000 × *g* for 10 s) and the supernatants (samples "S") were removed and kept on ice. After washing the beads four times with 1 ml of buffer A containing 0.1% (w/v) digitonin, immunoprecipitated proteins were eluted with 150 μl of Laemmli sample buffer [22] at 85 °C for 10 min. Fifteen microliters of 1 M dithiothreitol were subsequently added to the eluate. Samples "T" and "S" were subjected to



precipitation with trichloroacetic acid before SDS-PAGE analysis.

2.6. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS of tryptic digests from protein-containing polyacrylamide gel slices was carried out using a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). Samples were digested as described [23] and analyzed by HPLC-MS using a Discovery Bio Wide Pore C18, 5 mm (15 cm × 0.32 mm) HPLC column (Supelco, Bellefonte, USA). Proteins were identified by search with peptide-mass fingerprinting data from mass spectrometry on Protein Prospector [24].

2.7. Miscellaneous

Proteins were quantified by the Lowry method, using albumin as standard [25]. SDS-PAGE analysis was performed in 0.75-mm-thick, 11% polyacrylamide gels using the Laemmli discontinuous buffer system [22]. Silver staining of polyacrylamide gels was performed as described [26]. Western blotting onto nitrocellulose membranes was done as described by the manufacturer (Schleicher & Schuell). The polyclonal antibodies directed to mouse ALDP (serum 1664 [27]) and ALDPR (serum 7373 [28]) were used in immunoblotting analysis at 1:1000 (v/v) dilution. The rabbit antibody directed to rat PMP70 (Zymed Laboratories) and the mouse anti-α subunit of the mitochondrial ATPase complex (Molecular Probes) were used in immunoblotting analysis at 1:2000 (v/v) dilution. The primary antibodies were detected with a horseradish peroxidase conjugated donkey antirabbit or sheep antimouse antibodies (Amersham Biosciences) using ECL super signal West-dura substrates (Pierce).

Control and antimouse ALDP IgGs were isolated using protein A-Sepharose according to the manufacturer's instructions (Amersham Biosciences). The isolated IgGs were coupled to CNBr-activated Sepharose 4B beads at a ratio of 200 μg of protein per 12 mg of resin following the manufacturer's recommendations (Amersham Biosciences).

Densitometric analysis of Western blots was performed using the UN-SCAN-IT automated digitizing system.

3. Results

3.1. Mouse liver PMP70, ALDP and ALDPR behave as large protein complexes upon solubilization with digitonin

As a first step to characterize the quaternary structure of peroxisomal half ABC transporters, a preliminary set of experiments was performed to find the best solubilization conditions. These experiments were carried out taking into consideration two variables: (1) high yield in the solubilization procedure, so that the extracted protein population is representative of the population that exists in vivo and (2) mildness of the solubilization step to avoid disrupting protein–protein interactions. The first variable was quantified by densitometric analysis of Western blots containing known amounts of total peroxisomal proteins and the corresponding solubilized protein fractions; yields of 80% were routinely obtained (compare lanes Per and T in Fig. 3). The second variable was empirically assessed by subjecting solubilized proteins to centrifugation sedimentation analysis (see below).

For practical reasons, related to antibody availability and facility in obtaining highly purified peroxisomal fractions, these studies were performed with mouse liver proteins. The results of one of these experiments are shown in Fig. 1A. After solubilization of purified mouse liver peroxisomes using the mild detergent digitonin, PMP70, ALDP and ALDPR were found as large protein complexes (apparent molecular mass of 300–400 kDa) upon sucrose gradient centrifugation. Considering that all these proteins have a predicted molecular mass around 70 kDa, the estimated molecular masses may appear too large even if we assume that all these proteins are indeed in a dimeric state. However, it should be noted that the sedimentation properties of a solubilized membrane protein also depend on its partial specific volume, shape and amount and type of protein-bound detergents/membrane lipids. Thus, no conclusions regarding the true size of the observed protein complexes can be inferred from these experiments. In fact, by the same line of reasoning, it could be argued that the solubilized proteins are in the monomeric state. However, this is clearly not the case as shown by the following experiment. Digitonin-solubilized peroxisomal proteins were incubated in the

Fig. 2. Isolation of the PMP70-containing protein complex. (A) Digitonin-solubilized peroxisomal proteins (lane S, derived from 2 μg of starting material) were subjected to an ATP-agarose affinity chromatography. Eluted proteins (lane E, derived from 8 μg of peroxisomal protein) were loaded onto a sucrose gradient and centrifuged. The PMP70-enriched fraction (fraction 4 of the sucrose gradient; see below) was selected (lane F; derived from 80 μg of peroxisomal protein). Lane T, 2 μg of peroxisomal protein. Lane MW, molecular mass markers. A silver-stained polyacrylamide gel is shown. (B) ATP-agarose affinity chromatography eluted proteins (derived from 250 μg of peroxisomal proteins) were subjected to sedimentation analysis. The upper panel shows a silver-stained gel and the lower panel a Western blotting using the PMP70, ALDP and ALDPR antibodies. The identity of several proteins was determined by ESI-MS (see also Table 1): a—mitochondrial ATP-synthase α-chain, a subunit of the 750 kDa complex V [34]; b—PMP70; c—peroxisomal D-bifunctional protein, a homodimer of 154 kDa [35]; d—peroxisomal L-bifunctional protein, a 79-kDa protein monomeric in its native state [36]; e—urate oxidase (probably the trimeric form [37]). Some other proteins can be easily identified considering their abundance, molecular mass and subunit composition: f1, f2 and f3—72, 52 and 21 kDa subunits of palmitoyl-CoA oxidase, respectively, a dimeric protein with a native molecular mass of 150 kDa [38]; g—catalase, a tetrameric complex of 240 kDa [39]. Arrows A and B on the left indicate the expected migration positions of ALDP and ALDPR, respectively. The positions of the molecular mass standards (kDa) are indicated.

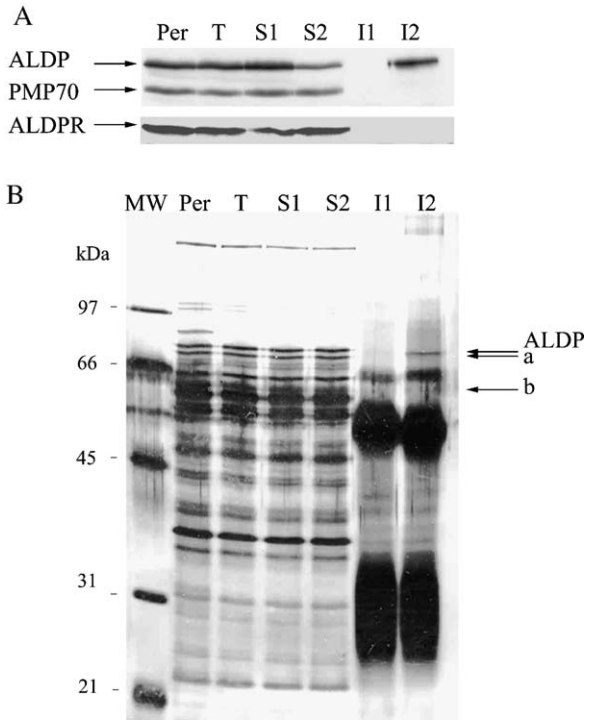


Fig. 3. Characterization of the ALDP-containing protein complex. Digitonin-solubilized peroxisomal proteins (lane T) were incubated with CNBr-activated Sepharose 4B beads previously coupled to control or anti-ALDP IgGs (see Materials and methods). After centrifugation, the supernatants were recovered, the beads were washed and the immunoprecipitated proteins were eluted with Laemmli sample buffer. Lanes S1 and I1: supernatant and immunoprecipitated proteins, respectively, obtained with control IgGs. Lanes S2 and I2: supernatant and immunoprecipitated proteins obtained with anti-ALDP IgGs, respectively. The protein samples were subjected to Western blotting using antibodies directed to ALDP, ALDPR and PMP70 (panel A) or analyzed by SDS-PAGE followed by silver staining (panel B). In panel A, protein loading was the following: lanes T, S1 and S2—proteins derived from 70 μg of peroxisomal protein; lanes I1 and I2—immunoprecipitated proteins derived from 140 μg of peroxisomal protein; lane Per: 70 μg of peroxisomal protein. In panel B, aliquots derived from 2 μg of peroxisomal protein were loaded in lanes T, S1 and S2. Lanes I1 and I2 contain immunoprecipitated proteins from 140 μg of peroxisomal protein. Lane Per—2 μg of peroxisomal proteins. Arrows a and b indicate the expected migration positions of ALDPR and PMP70, respectively. Molecular mass markers are shown on the left-hand lane (lane MW).

presence of urea, a chaotropic agent frequently used to disrupt protein–protein interactions. After diluting the solution with digitonin-containing buffer (see Materials and methods), the protein sample was subjected to sedimentation analysis. Although a fraction of several peroxisomal proteins precipitated after this treatment and were found at the bottom of the centrifuge tube (data not shown), a small amount of PMP70 and ALDP remained soluble. Two different populations of PMP70 were detected in this experiment (see Fig. 1B): a minor fraction of PMP70 resisted to the urea treatment in an apparently intact conformation (fraction 4 of the gradient); the majority of soluble PMP70, however, was found in fractions 7–8 of the sucrose gradient (apparent molecular mass of 100–150 kDa). Similar results were obtained for ALDP, although in this case the vast majority of the protein was detected in fraction 7–8 of the sucrose gradient (see Fig. 1B). These data taken together indicate that under the solubilization conditions employed, peroxisomal transporters are indeed components of protein complexes.

3.2. Purification of mouse liver PMP70

As shown above, digitonin-solubilized PMP70, ALDP and ALDPR behave as 300–400 kDa protein complexes upon centrifugation sedimentation analysis. Such apparent molecular mass could be explained by assuming that these proteins are dimers that bind large amounts of detergent (see Discussion). Alternatively, this behavior could reflect the existence of larger protein complexes comprising, in addition to the transporters, other proteins. Examples of ABC transporter protein complexes containing accessory proteins are abundant (e.g., tapasin and the TAP transporter of mammals [29]) or the maltose binding protein and the ABC maltose transporter of *Escherichia coli*; reviewed in Ref. [30]).

To clarify this issue, the PMP70-containing protein complex was purified. For this purpose, a simple two-step procedure was developed. In the first step, digitonin-solubilized peroxisomal proteins were subjected to an ATP-agarose affinity chromatography (see Materials and methods for details). Western blotting analysis revealed that more than 90% of the amount of PMP70 applied to the affinity matrix was recovered after this step (data not shown). Interestingly, several other proteins were specifically eluted from the ATP-agarose matrix (see Figs. 2A and B and Table 1). Although some of these proteins may indeed bind ATP (this is surely the case for mitochondrial complex V; reviewed in Ref. [31]), the majority of the proteins identified bind adenine-containing molecules (e.g., NADP, FAD, NAD), but not ATP itself (see legend to Fig. 2). In the second step of the purification procedure, the high sedimen-

Table 1
Identification of ATP-agarose affinity chromatography purified proteins using ESI-MS

Protein band	Protein name	MW (Da)	Mean error (ppm)	Sequence coverage (%)	MOWSE score[a]
A	ATP synthase alpha chain	59,753	8.9	32.0	1.216e+008
B	PMP70	75,483	−15.1	19.0	4.619e+007
C	Peroxisomal D-bifunctional enzyme	79,525	−3.25	18.0	1.928e+007
D	Peroxisomal L-bifunctional enzyme	78,244	0.534	22.0	1.896e+007
E	Urate oxidase	35,039	14.0	32.0	5.289e+006

[a] The MOWSE score reported by MS-Fit is based on the scoring system described in Ref. [40].

tation coefficient of digitonin-solubilized PMP70 was explored. As shown in Fig. 2A, a homogeneous PMP70 preparation was obtained: no stoichiometric amounts of other proteins co-purifying with PMP70 could be detected upon SDS-PAGE analysis. Furthermore, ESI-MS analysis of the protein band containing PMP70 failed to reveal the presence of any protein other than PMP70 itself. We conclude that the majority of mouse liver PMP70 is a homomeric protein.

It may be interesting to note that high amounts of PMP70 (5 μg/mg of total peroxisomal protein) can be easily obtained using the method described here. Biochemical experiments to further characterize the structure/function of this protein are under progress.

3.3. Characterization of the mouse liver ALDP protein complex

In rat liver, ALDP and ALDPR are much less abundant proteins than PMP70 [11]. This is probably also the case for mouse liver ALDP and ALDPR. Indeed, although these two mouse proteins are also retained in an ATP-agarose matrix, as revealed by Western blot analysis (see Fig. 2B), no protein band corresponding to either of these proteins can be detected in the polyacrylamide gels shown in Fig. 2. Thus, to characterize the quaternary structure of ALDP, a different strategy was used. Digitonin-solubilized mouse liver peroxisomes were subjected to an immunoprecipitation experiment using the anti-ALDP antibody. Immunoprecipitated proteins were then analyzed by Western blotting using antibodies directed to ALDP, PMP70 and ALDPR. As shown in Fig. 3A, about 80% of the ALDP protein was immunoprecipitated by the anti-ALDP antibody. Most importantly, neither PMP70 nor ALDPR could be detected in the immunoprecipitate.

To corroborate these observations, the immunoprecipitated proteins were also subjected to SDS-PAGE and stained with silver. As shown in Fig. 3B, besides antibody-derived protein bands, only one protein band displaying exactly the electrophoretic behavior of ALDP is visible in the immunoprecipitate obtained with the anti-ALDP antibody. Taken together, these data strongly suggest that the majority of mouse liver ALDP (if not all) is a homomeric protein assembly.

4. Discussion

The most critical step in any protocol aiming at the purification of a membrane protein resides in the solubilization procedure. Solubilization conditions have to ensure high yields in the extraction of the protein(s) under study, and, if the aim is to isolate protein complexes, they have to be gentle enough so that protein–protein interactions are preserved. When determining the best solubilization conditions for a given protein it is important to define the variable "solubility". In general terms, a soluble protein should remain in the supernatant after centrifugation for 1 h at $105\,000 \times g$ or should elute in the included volume from a gel filtration medium with very large pores such as Sepharose 4B [32]. In this work, we used the former criterion. Unfortunately, these considerations are sometimes overlooked and it is not uncommon to find in the literature experiments involving membrane proteins in which the detergent extract was just subjected to a low speed centrifugation (e.g., 5 min at $15\,000 \times g$) before immunoprecipitation. The risk associated with this type of procedure is high: If the protein under study is not completely in solution, then it is likely that other insoluble proteins will be co-immunoprecipitated not because they are truly associated with the protein of interest but because they reside in the same membrane fragments. This is probably the reason why the data reported here are so different from recent results suggesting that rat liver PMP70 and ALDP are components of the same protein assembly [11].

The aim of the work presented here was to determine the type of protein–protein interactions in which peroxisomal half ABC transporters are involved in vivo. In particular, we were interested in assessing whether these transporter proteins can engage in heteromeric interactions with each other and/or with other unrelated proteins under normal expression conditions. It should be noted that data suggesting that all these peroxisomal ABC transporters interact with each other have already been described. Indeed, pairwise interactions involving PMP70, ALDP and ALPR were observed when using a combination of yeast two-hybrid assays and Western blotting analysis of immunoprecipitates obtained from cultured cells overexpressing epitope-tagged peroxisomal transporters [9]. While these results suggest that these proteins have the potential to interact with each other forming both homo- and heterodimers, the nonquantitative nature of these techniques does not allow us to infer which kind of interactions (if any) prevail in vivo.

Considering the unexpectedly high apparent molecular mass of digitonin-solubilized peroxisomal ABC transporters (300–400 kDa), we started our studies by addressing the possibility that these transporters interact with other nonrelated proteins. For this purpose, we isolated chemical amounts of the PMP70-containing complex. Our results clearly show that no other protein in stoichiometric amounts was co-purified with PMP70. Thus, the strikingly high sedimentation coefficient of PMP70 (and of the other two ABC transporters characterized in this work) is an intrinsic property of these digitonin-solubilized proteins. What is the reason for such high sedimentation coefficients? The most plausible explanation is to assume that all these proteins are dimers (approximate molecular masses of 140 kDa) that bind an amount of detergent that equals 100–150% of their masses (140–210 kDa). Detergent/protein ratios of 1:1.5 (w/w) are quite common for solubilized hydrophobic proteins (e.g., Ref. [33]). The fact that the majority of urea-treated PMP70 and ALDP (presumably representing mono-

meric protein) display an apparent molecular mass of 100–150 kDa supports this interpretation. However, it has to be emphasized that further experiments are necessary to confirm this hypothesis. Thus, at this moment, other possibilities (e.g., a homotetrameric structure), although unlikely, cannot be formally excluded.

To assess whether peroxisomal ABC transporters are capable of forming heterodimers, we focused our attention on ALDP. To characterize the quaternary structure of this protein, we performed immunoprecipitation experiments using mouse liver peroxisomes. Western blotting analysis clearly show that neither PMP70 nor ALDPR could be co-immunoprecipitated with ALDP. Silver staining of a polyacrylamide gel containing the immunoprecipitated proteins confirmed this observation: No protein band other than the one corresponding to ALDP could be observed. Thus, the vast majority (if not all) of mouse liver ALDP is a homomeric protein.

Due to the fact that antibodies directed to mouse PMP70R are not yet available, the properties of this protein regarding its native molecular mass and interactions with the other three peroxisomal half ABC transporters could not be assessed in this work. Although there are no data whatsoever to support the existence of heteromeric interactions involving PMP70R, our data do not allow us to exclude this possibility. Indeed, PMP70R is much less abundant than PMP70 and ALDP (less than 1/70 and 1/10, respectively [11]) and thus, the presence of PMP70R in our silver-stained gels would not be noted. Further work is necessary to clarify this issue.

In conclusion, our results indicate that mouse liver PMP70 and ALDP are mostly (if not exclusively) homomeric protein assemblies. Determining whether this observation can be extended to other mouse organs and to other organisms, humans in particular, is of main importance to understand the pathophysiology of X-ALD.

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